Repetition of Ischemic Preconditioning Augments Endothelium-Dependent Vasodilation in Humans
Role of Endothelium-Derived Nitric Oxide and Endothelial Progenitor Cells

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Background—Several studies have shown that both early and late effects of ischemic preconditioning (IPC) protect against myocardial injury after ischemic reperfusion.

Methods and Results—The purpose of this study was to evaluate the late effects of IPC on endothelial function in humans. Late phase of IPC was induced by upper limb ischemia (cuff inflation of over 200 mm Hg for 5 minutes) 6 times a day for 1 month. We evaluated forearm blood flow (FBF) responses to acetylcholine (ACh) and to sodium nitroprusside (SNP) before and after IPC stimulus in 30 young healthy men. FBF was measured using a strain-gauge plethysmograph. The IPC stimulus significantly increased plasma concentration of vascular endothelial growth factor (VEGF), circulating level of endothelial progenitor cells (EPCs), and FBF responses to ACh, but these did not change in the control group. The FBF responses to SNP were similar before and after the IPC stimulus. Infusion of N\textsuperscript{G}-monomethyl-L-arginine, a nitric oxide synthase inhibitor, completely eliminated the IPC stimulus-induced augmentation of FBF responses to ACh. In the cotralateral arms of subjects that received the IPC stimulus, FBF responses to ACh did not change, but levels of VEGF and circulating EPCs increased.

Conclusions—These findings suggest that repetition of late IPC stimulus augments endothelium-dependent vasodilation in humans through increases in nitric oxide production and number of EPCs under a local condition. Repetition of IPC stimulus may be a simple, safe, and feasible therapeutic technique for endothelial protection of peripheral vessels.

Key Words: preconditioning ■ endothelial function ■ nitric oxide ■ vascular endothelial growth factor ■ endothelial progenitor cells

A brief ischemic period followed by episodes of reperfusion increase the resistance to further ischemic damage. This phenomenon is called ischemic preconditioning (IPC). IPC has been observed in the heart and other organs.\textsuperscript{1–3} IPC is an important mechanism by which tissues protect themselves from an impending accident. A minimum of 2 minutes of ischemia and reperfusion for the human myocardium is required to induce an immediate protective effect of IPC.\textsuperscript{1–3} IPC occurs in 2 phases: an “early” phase of protection, which develops within minutes after initial ischemia and lasts 2 to 3 hours, and a “late” phase of protection, which begins 12 to 24 later and lasts 3 to 4 days.\textsuperscript{4} Late IPC has protective effects against myocardial infarction and myocardial stunning, but early IPC only has an effect against myocardial infarction.\textsuperscript{5–9} Several studies have shown that prodromal angina pectoris occurring shortly before the onset of infarction reduced infarct size and improved left ventricular function.\textsuperscript{10,11} This phenomenon is associated with beneficial effects of late IPC but not early IPC. Therefore, clinically, late phase of IPC repetition has greater significance for cardiovascular protection.

IPC is thought to have components of endothelium-derived nitric oxide (NO) and adenosine and to be multifactorial. Endothelial function, especially NO, plays a critical role in the development and maintenance of cardiovascular diseases.\textsuperscript{12–17} Therefore, from a clinical perspective, it is important to select an appropriate intervention that is effective in improving endothelial dysfunction in cardiovascular diseases. It is postulated that under the condition of hypoxia, vascular endothelial growth factor (VEGF) gene expression is upregulated by induction of hypoxia-inducible factor-1 (HIF-1), resulting in increase in the number of endothelial progenitor cells (EPCs). Interestingly, endothelial function has been found to be associated with the number of circulating...
EPCs in humans.\textsuperscript{18} It is also thought that IPC (IPC-induced hypoxia) augments endothelial function through an increase in EPCs.

Most studies on preconditioning have focused on the ability to protect myocytes, and there is little information on the role of IPC in endothelium. To determine the effects of late phase and repetition of IPC on vascular function in humans, we measured vascular responses to acetylcholine (ACh), an endothelium-dependent vasodilator, and sodium nitroprusside (SNP), an endothelium-independent vasodilator, and circulating levels of EPCs in healthy young men.

**Methods**

**Subjects**

We studied 20 young healthy men (mean age, 28.1 ± 3.9 years) who had no history of cardiovascular or cerebrovascular disease, hypertension, hypercholesterolemia, diabetes mellitus, liver disease, renal disease, smoking habit, or other diseases. The subjects were divided randomly into the IPC group (n=10; mean age, 27.9 ± 4.0 years) and control group (n=10; mean age, 28.3 ± 3.6 years). The Ethical Committee of Hiroshima University Graduate School of Biomedical Sciences approved the study protocol. Written informed consent for participation in the study was obtained from all subjects.

In addition, we evaluated the effects of IPC on endothelial function of the contralateral arm in 10 young healthy men (mean age, 27.3 ± 2.4 0 years) using a protocol identical to that used for the study of IPC. These subjects were different from the subjects in the group in which endothelium-dependent and -independent vascular responses at baseline were compared.

**Study Protocol**

None of the subjects received any drugs for at least 24 hours before the study. An upper arm cuff was inflated to 200 mm Hg for 5 minutes 6 times a day for 4 weeks using a rapid cuff inflator (EC-20, Hokanson, Inc) to obtain repetition of transient ischemia as a strategy of IPC. Controls were subjected to 4 weeks of follow-up without any lifestyle modification. Forearm vascular responses to ACh (Daichi Pharmaceutical Co) and to SNP (Maruishi Pharma Co) in the contralateral arm or IPC-stimulated arm in subjects that received the IPC were evaluated before and after 4 weeks of IPC repetition stimulus. The studies began at 8:30 AM after 14 hours of the last IPC stimulus. Subjects were kept in a supine position in a quiet, dark, and air-conditioned room throughout the study. A 23-gauge catheter was inserted into the contralateral artery for infusion of using 1% lidocaine to exclude the hand circulation from the measurements. The upper arm cuff was inflated to 40 mm Hg for 7 seconds during each 15-second cycle using a rapid cuff inflator (EC-20, Hokanson, Inc) to occlude venous outflow from the arm. The FBF output signal was transmitted to a recorder (U-228, Advance Co). FBF is expressed as mL per minute per 100 mL of forearm tissue. Four plethysmographic measurements were averaged to yield values for FBF at baseline and during the administration of drugs. FBF was calculated by 2 observers blinded to the experimental protocol from the linear portions of the plethysmographic recordings. The intraobserver coefficient of variation was 5.7%.

**Measurement of EPCs**

The number of EPCs was analyzed by flow cytometry as previously described.\textsuperscript{18} Briefly, samples of venous blood were placed in tubes containing sodium EDTA (7 mg/mL) and in polystyrene tubes. The EDTA-containing tubes were chilled promptly in an ice bath. Peripheral blood mononuclear cells were immediately isolated by Ficoll density gradient centrifugation (AXIS-SHIELD). After thawing, 1 × 10\textsuperscript{6} peripheral blood mononuclear cells were incubated for 10 minutes with monoclonal antibodies against human fluorescein isothiocyanate (FITC)-conjugated anti-CD45 (Miltenyi Biotec), PE-conjugated anti-AC133 (Miltenyi Biotec), and activated protein C isothiocyanate (FITC)-conjugated anti-CD45 (Miltenyi Biotec), and activated protein C (APC)-conjugated anti-CD34 monoclonal antibody (Becton Dickinson Biosciences). To assess background, isotype controls were used as negative controls based on the species and IgG subclass of each antibody. After incubation, erythrocytes were lysed, and the remaining cells were washed with phosphate-buffered saline, fixed in 2% paraformaldehyde, and analyzed on a fluorescence-activated-cell sorter (FACS) Calibur Flow Cytometer (Becton Dickinson Biosciences). Each analysis consisted of 500,000 events. To quantify the amount of CD34\textsuperscript{+}ACC133\textsuperscript{+}CD45\textsuperscript{+} cells, the mononuclear cell fraction was gated and analyzed for the expression of AC133 and CD45. Only the AC133\textsuperscript{+}CD45\textsuperscript{+} cells finally investigated for the count of CD34\textsuperscript{+} cells.

**Analytical Methods**

Samples of venous blood were placed in tubes containing sodium EDTA (1 mg/mL) and in polystyrene tubes. The EDTA-containing tubes were chilled promptly in an ice bath. Samples were stored at −80°C until the time of assay. Plasma concentrations of VEGF were measured using ELISA kits (R&D Systems Co). Serum concentrations of total cholesterol, triglycerides, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, glucose, and electrolytes were determined by routine chemical methods. Serum insulin was measured using an automated radioimmunoassay technique.

**Statistical Methods**

Values are expressed as the mean ± SE. The Mann-Whitney U test was used to evaluate differences between before and after IPC stimulus with respect to baseline parameters. Two-tailed Student paired t test was used to evaluate differences before and after IPC stimulus. The FBF responses to ACh and SNP before and after IPC stimulus were analyzed by two-way ANOVA for repeated measures, followed by Scheffe F test. Results were considered significant at P < 0.05.

**Results**

**Clinical Characteristics**

The baseline clinical characteristics of the 10 control subjects at 0 weeks and 4 weeks, the 10 subjects in whom data were
significant change was observed in arterial blood pressure and at the end of the 4-week study period in both the IPC and SNP during infusion of SNP were similar at the beginning or in the contralateral group (Figure 1B). The increases in 4 week follow-up period in the control group (Figure 1A)

Intraarterial infusion of L-NMMA significantly decreased presence of L-NMMA.

Effects of IPC on FBF Responses to ACh in the Effects of IPC on FBF Responses to ACh in the 3 groups at both time points (Figure 3A, 3B, and 3C). The intraarterial infusion of L-NMMA decreased the response to ACh in the 3 groups at both time points (Figure 3A, 3B, and 3C). The intraarterial infusion of L-NMMA abolished the augmentation of FBF response to ACh by IPC stimulus (Figure 3C). Neither arterial blood pressure nor heart rate was significantly changed by intraarterial infusion of ACh in the presence of L-NMMA.

Effects of IPC on EPCs
IPC stimulus for 4 weeks increased the number of circulating EPCs from 1373±178 to 1701±183 mL/min/100 mL tissue (P<0.05) in the contralateral group and from 1394±169 to 1659±161 mL (P<0.05) in the IPC group, whereas there was no significant difference between the number of circulating EPCs at 0 weeks and that at 4 weeks in the control group (Figure 4). The numbers of EPCs were similar in the contralateral group and IPC group before and after IPC stimulus. The increase in maximal FBF response to ACh correlated with the increase in number of circulating EPCs (r=0.69, P<0.001) in the IPC group (Figure 5) but not in the contralateral group. There was no correlation between increase in number of circulating EPCs and increase in plasma VEGF concentration. No correlation was found between increase in maximal FBF response to ACh and changes in blood pressure, heart rate, VEGF, or other variables or obtained from the contralateral arm using a protocol identical to that used for the study of IPC before (0 weeks) and after (4 weeks) IPC, and the 10 subjects before (0 weeks) and after (4 weeks) IPC are summarized in the Table. The plasma concentration of VEGF was increased significantly from 88.1±18.8 to 118.1±11.5 pg/mL (P<0.05) in the contralateral group and from 4.9±0.6 mL/min/100 mL tissue (P<0.05) in the IPC group by 4 weeks of IPC but was not altered in the 4-week follow-up period in the control group.

IPC stimulus did not alter systemic hemodynamics such as blood pressure, heart rate, and lipid profile. Other parameters before and after IPC stimulus were similar. In the control group, the baseline clinical characteristics were similar at 0 weeks and 4 weeks of follow-up.

Effects of IPC on FBF Responses to ACh and SNP
IPC stimulus did not alter baseline FBF in the contralateral group or IPC group. The response of FBF to infusion of ACh was increased significantly from 18.3±0.9 to 24.8±1.9 mL/min/100 mL tissue (P<0.05) by 4 weeks of IPC in the IPC group (Figure 1C) but was not altered in the 4 week follow-up period in the control group (Figure 1A) or in the contralateral group (Figure 1B). The increases in FBF during infusion of SNP were similar at the beginning and at the end of the 4-week study period in both the IPC groups and the control group (Figure 2A, 2B, and 2C). No significant change was observed in arterial blood pressure or heart rate with intra-arterial infusion of ACh and SNP.

Effects of IPC on FBF Responses to ACh in the Presence of L-NMMA
Intraarterial infusion of L-NMMA significantly decreased baseline FBF from 4.7±0.5 to 4.1±0.6 mL/min/100 mL tissue (P<0.05) in the contralateral group and from 4.9±0.4 to 4.0±0.6 mL/min/100 mL tissue (P<0.05) in the IPC group before IPC stimulus and from 4.8±0.6 to 4.1±0.6 mL/min/100 mL tissue (P<0.05) in the contralateral group and from 5.2±0.6 to 4.5±0.8 mL/min/100 mL tissue (P<0.05) in the IPC group after IPC stimulus. Changes in basal forearm vascular responses to L-NMMA were similar in the 3 groups at 0 weeks and 4 weeks (Figure 3A, 3B, and 3C). The intraarterial infusion of L-NMMA decreased the response to ACh in the 3 groups at both time points (Figure 3A, 3B, and 3C). The intraarterial infusion of L-NMMA abolished the augmentation of FBF response to ACh by IPC stimulus (Figure 3C). Neither arterial blood pressure nor heart rate was significantly changed by intraarterial infusion of ACh in the presence of L-NMMA.
Discussion
Repetition of IPC stimulus over a 4-week period augmented endothelium-dependent vasodilation but not endothelium-independent vasodilation in healthy subjects. L-NMMA eliminated the IPC stimulus-induced augmentation of FBF response to ACh. In addition, the increase in maximal FBF response to ACh correlated with the increase in the number of EPCs after repetition of IPC. These findings suggest that the augmentation of ACh-induced vasodilation may be related to an improvement in the function of the endothelium, but not vascular smooth muscle, and may be attributable to an increase in NO production through, at least in part, an increase in EPCs.

We selected healthy young men to avoid the possibility of alteration in endothelial function and number of EPCs caused by factors such as hypertension, heart failure, atherosclerosis, hypercholesterolemia, diabetes mellitus, smoking, aging, and menstrual cycle.

Cardiovascular protection afforded by the early phase of IPC has been shown to be mediated by stimulation of receptors linked to protein kinase C (PKC) activation by adenosine, bradykinin, NO, and free radicals. PKC is a trigger of the opening of ATP-sensitive potassium channels, leading to cardiovascular protection. Several investigators have shown that early IPC prevents damage to organs subjected to ischemic reperfusion injury in animal models. Recently, Kharbanda et al reported that short periods of limb ischemia (early IPC) might have the potential to reduce endothelial injury during ischemic reperfusion in humans. In the present study, repetition of brief IPC, as a late phase of IPC, was evaluated because the effects of late phase and repetition of IPC on endothelial function in humans remain
unclear. In addition, the precise mechanisms of late IPC in humans is still unclear.

There are several possible explanations for the IPC stimulus-induced augmentation of forearm vascular response to ACh in humans. Several lines of evidence have shown that the late effect of IPC is mainly attributable to an increase in NO production.4,6,21 In the present study, L-NMMA completely abolished the IPC stimulus-induced augmentation of FBF responses to ACh. In a recent study, the nonselective NOS inhibitor N^o^-nitro-L-arginine, but not the inducible NOS inhibitor 1400W, completely eliminated the protective effects of late phase of IPC against coronary endothelial injury.22 Bolli et al23 proposed that NO plays a prominent role in initiating the late phase of IPC. These findings suggest that the beneficial effects of IPC repetition are due to activation of eNOS, resulting in increased NO production.

One possible mechanism by which repetition of IPC augments endothelial function is an increase in vascular shear stress resulting from increased blood flow. Acute or chronic increases in shear stress stimulate the release of NO in isolated vessels and cultured cells.24,25 Sessa et al26 demonstrated that the increase in shear stress in epicardial coronary arteries of dogs for 10 days of treadmill exercise enhanced the expression of the vascular eNOS gene, leading to ACh-stimulated NO release. The upregulation of eNOS mRNA levels and eNOS protein levels by repetition of IPC may contribute to improvement in endothelial function through an increase in NO production. In addition, chronic increases in shear stress have been shown to lead to functional and histological alterations of the vascular endothelium, resulting in enhanced vascular structure and function.27 This beneficial change in the endothelium after repetition of IPC also may contribute to the augmented forearm vascular response to ACh and to the ACh-stimulated NO release.

Repetition of IPC, probably by an increase in shear stress, exerts its beneficial effects on endothelial function by activation of several signal transduction pathways.28,29 It is thought that mechanosensors, such as caveolae, G proteins, ion channels, and integrins on the membranes of endothelial cells sense shear stress and transduce stimuli into biochemical signals, and then several stimuli activate Ras/Raf/MEK/extracellular signal regulated kinase (ERK) and c-Src pathways, leading to an increase in eNOS activity.28,30,31

Several lines of evidence have indicated that hypoxia per se enhances VEGF gene expression.32,33 It is well known that VEGF gene expression is upregulated by HIF-1 under the condition of hypoxia.34 HIF-1 is a heterodimer composed of 2 subunits, HIF-1 alpha and HIF-1 beta, and promotes transcription by combining with hypoxia response element in its target gene.34 In the present study, repetition of IPC increased plasma VEGF levels and the number of EPCs. Wang et al5 reported a significant increase in the number of functional capillaries and arteriole diameter in rats 24 hours after ischemic reperfusion. These findings suggest that the hypoxia–HIF-1–VEGF pathway may play an important role in IPC-induced angiogenesis in skeletal muscle. It is proposed the model of VEGF-modulating–eNOS activation by repetition of IPC: IPC causes hypoxia in skeletal muscle; HIF-1 upregulates expression of the VEGF gene; VEGF activates the PI3K/Akt pathway; calcium-calmodulin together with heat shock protein 90 (HSP90) displaces eNOS from caveolin-1; the interaction of HSP90 with Akt and eNOS permits HSP90 to serve as a docking site for phosphorylation of eNOS Ser1177 by Akt; HSP90 stabilizes the binding of calmodulin and eNOS; electron flux is caused from the reductase to the oxygenase domain of eNOS; and NO is released from L-arginine in endothelial cells.
to an increase in capillary density, resulting in augmentation of endothelial function through an increase in NO production.

Interestingly, endothelium-dependent vasodilation did not change after 4 weeks of IPC in the contralateral arm in subjects that received the preconditioning protocol, whereas the plasma concentration of VEGF and the number of EPCs increased after 4 weeks of IPC in the contralateral arm. Although the precise mechanisms by which increases in circulating VEGF and EPCs do not induce the augmentation of endothelium-dependent vasodilation in the contralateral arm that did not receive direct IPC stimulus remain unclear, our results suggest that IPC enhances endothelial function through local effects.

In a rabbit model of chronic hindlimb ischemia, intravenous administration of 3 mg/kg VEGF increased muscle blood flow and intraarterial administration of 1 mg VEGF...
stimulated muscle function. 36 Unfortunately, we do not have VEGF for human use for intraarterial administration. There are no data showing that detected elevation of VEGF observed in the present study is sufficient to activate eNOS gene expression and activity. In the present study, increase in circulating VEGF did not induce the augmentation of ACh-induced vasodilation in the contralateral arm that did not receive direct IPC stimulus. It is unlikely that the elevation of VEGF detected in the present study induces augmentation of endothelium-dependent vasodilation.

Green et al 37 reported that exercise training enhanced reactive hyperemic response, as an index of vascular structure, in the preferred arm but not in nonpreferred arm without influencing basal or ACh-induced vasodilation in tennis players. Although the discrepancy in the results of the present study may be attributable to the different stimulus, aerobic exercise and IPC, we cannot deny the possibility that repletion of IPC stimulus alters vascular structure per se. A reactive hyperemia measurement of peak flow might be helpful in future studies to show structural changes in forearm vessels before and after IPC stimulus.

In conclusion, repetition of IPC augmented endothelial function through an increase in NO production. Endothelial dysfunction is the initial step in the pathogenesis of atherosclerosis, resulting in cardiovascular complications. It is important to select an appropriate intervention that is effective in improving or augmenting endothelial function. Repetition of IPC may be a simple, safe, and feasible therapeutic technique for endothelial protection of peripheral vessels. Furthermore, this technique has the potential for improving endothelial function as a new treatment for cardiovascular disease associated with endothelial dysfunction.

Disclosures

None.

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